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Biochimica et Biophysica Acta 1452 (1999) 145–150

BIOCHIMICA ET BIOPHYSICA ACTA

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Adenine incorporation in human and rat endothelium

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Received 27 May 1999; received in revised form 23 August 1999; accepted 24 August 1999

Abstract

Adenine (ADE) reutilisation is an important pathway of adenylate pool regeneration. Data on the rate of this process in different types of cells, its regulation and the importance of species differences is limited. In this study we evaluated adenine incorporation rate and the effect of metabolic factors on this process in human and rat endothelium and compared it to adenine phosphoribosyltransferase (APRT) activity. Microvascular endothelial cells from human (HE) and rat (RE) hearts and a transformed human microvascular endothelial cell line (HMEC-1) were investigated. The rate of adenine incorporation into the adenine nucleotide pool under control conditions was 3.1 ± 0.3, 82.8 ± 11.1 and 115.1 ± 11.2 pmol/min per mg protein for HE, RE and HMEC-1, respectively. In the presence of 2.5 mM ribose or elevated inorganic phosphate concentration in the medium (4.8 mM), few changes were observed in all types of cells. In the presence of both ribose and high inorganic phosphate, the rate of adenine incorporation for RE and HMEC-1 was not significantly different from control, while in HE the rate of adenine incorporation into adenine nucleotides was increased by 75%. Activities of APRT in RE and HMEC-1 were 237.7 ± 23.2 and 262.0 ± 30.6 pmol/min per mg protein respectively while the activity in HE was markedly lower 48.7 ± 3.0 pmol/min per mg protein. In conclusion, nucleotide synthesis from adenine seems to be a slow process in human cardiac microvascular endothelium but it is fast and efficient in rat heart microvascular endothelial cells. Low APRT activity in normal human endothelial cells seems to be the most likely mechanism for this. However, adenine incorporation rate and APRT activity could be greatly enhanced in human endothelium, as demonstrated in transformed cells.

Keywords: Adenine; Adenosine triphosphate; Adenine nucleotide; Adenine phosphoribosyltransferase; (Heart); (Endothelium)

1. Introduction

Adenine (ADE) reutilisation is an important pathway of adenylate pool regeneration. Although the maximal rate of adenine phosphoribosylation is lower than adenosine phosphorylation in the heart, it is much faster than purine synthesis de novo or adenine nucleotide synthesis from hypoxanthine [1]. Adenine incorporation requires phosphoribosylpyrophosphate (PRPP). Endogenous PRPP synthesis is dependent on the pentose phosphate pathway but, alternatively, it could be produced from an exogenous supply of ribose. The presence of ribose was found to increase ATP synthesis in the heart together with enhancement of mechanical recovery after car-

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0167-4889/99/$ – see front matter
PII: S0167-4889(99)00122-6

dioplegia and ischaemia [2–4] but it has not been identified which types of cells are important in this process. The enzyme responsible for PRPP production, PRPP synthetase, is strongly activated by inorganic phosphate [5], which could be important in increasing the supply of PRPP for adenine reutilisation.

It has been shown that the adenine nucleotide pool size is exceptionally high in endothelial cells but the importance of this phenomenon for the function of endothelial cells is uncertain [6]. Endothelial ATP and nucleotide pool could be important not only for maintenance of cell viability but also for the physiological function of these cells. A mechanism for nucleotide release from endothelial cells has been demonstrated [7] which could be involved in the control of thrombosis and inflammation. Extracellular ATP was found to affect a number of metabolic and regulatory processes through several types of purinergic receptors [8]. Nucleotide levels could be important also in cytoprotective mechanisms, such as adenosine production. Although the contribution of endothelial cells for adenosine production is small under ischaemic conditions, this proportion under normoxia is considerable [9,10]. We have shown previously the association between nucleotide pool and adenosine production in perfused heart and in isolated cardiomyocytes [11,12].

The aim of the present study was the evaluation of the rate, concentration dependence and the activity of the enzyme responsible for adenine incorporation into the adenylate pool in human and rat capillary endothelium and in transformed human microvascular cell line. Furthermore, the effects of ribose and increased inorganic phosphate concentration on this process were studied in detail.

2. Materials and methods

2.1. Endothelial cell isolation and culture

All animals received humane care in compliance with the *Guide for the Care and Use of Laboratory Animals* published by the National Institutes of Health (NIH publication no. 85-23, revised 1985). Rat endothelial cells were cultured after collagenase digestion of the heart according to the procedure described by Piper et al. [13] as described in detail previously [11]. Endothelial cells were separated from cardiomyocytes by centrifugation and were plated initially in 6-cm diameter plastic culture dishes and then after 2–3 passages transferred into 3.5-cm diameter dishes. Human tissue was used in accordance with the ethical standards as formulated in the Helsinki Declaration of 1975 (revised in 1983).

Human endothelial cells were isolated from the left ventricle from the explanted heart. The method of McDouall et al. [14] was followed. Briefly, epicardium and endocardium were discarded to minimise contamination by other cell types, the tissue was digested with collagenase and endothelial cells were isolated by positive selection on HLA-DR and Ulex-coated Dynabeads.

Human derived endothelial cell line (HMEC-1) used in another series of experiments were obtained from human dermal microvascular cells transfected with the simian virus 40 large T antigen. This cell line has been shown immunologically to retain the characteristics of normal human microvascular endothelial cells [15]. Cultures of endothelial cells were used for experiments after reaching confluence.

2.2. Experimental protocol for measurement of adenine incorporation

Firstly, incubation medium was removed and replaced by Hank's balanced salt solution (HBSS). After two subsequent changes of HBSS, a final volume of 1.2 ml was added and the experiment was started. The initial pre-incubation time was 30 min after which [8-^{14}C]adenine was added at a concentration varying from 0.3 μM to 0.3 mM as indicated. Ribose was added or inorganic phosphate concentration increased for the entire 30 min of pre-incubation. Incubation with adenine was carried out for 10 min (for human cells) or for 5 min (for rat cells). At the end of the incubation, the medium was collected and 0.6 ml of cold 0.4 M $HClO_4$ added to the incubation dish to extract cellular nucleotides. Acid extracts were centrifuged and neutralised with 2 M KOH followed by centrifugation. Supernatants were subjected to further analysis by HPLC as described below. Protein concentration was evaluated using the Bradford method [16] after dissolving the perchloric acid precipitates with 0.5 M NaOH.

2.3. Adenine phosphoribosyltransferase (APRT) assay procedure

Cell homogenates for enzyme assay were prepared from confluent endothelial cells grown in 25-cm^2 flasks. Culture media was removed and cells were washed three times with phosphate-buffered saline (PBS). Homogenisation buffer containing 150 mM KCl, 20 mM Tris, 1 mM EDTA, 1 mM dithiothreitol (pH 7.0) plus 0.1% Triton X-100 (0.1 ml) was added in three portions to maximise cell recovery. Endothelial cell homogenates were diluted with incubation buffer before assay. The incubation buffer consisted of 50 mM imidazole and 10 mM $MgCl_2$ (pH 7.0). The reaction was initiated by the addition of 20 μl of diluted cell extract to an equal volume of substrate solution, incubated at 37°C for 10 min and terminated by the addition of 20 μl of 1.3 M $HClO_4$. The substrate solution contained 8 mM PRPP, 200 μM methylene adenosine 5′-diphosphate (AOPCP), 200 μM [8-^{14}C]adenine with a specific activity of 12.5 μCi/μmol, all dissolved in incubation buffer. For blanks, perchloric acid was added to the substrate solution prior to the addition of cell extract. Samples were neutralised with 7 μl of 3 M K_3PO_4 and centrifuged at 13 000 rpm for 3 min. The resultant supernatant was analysed for conversion of substrate to products by collection of radioactive peaks separated by reverse-phase HPLC [17]. The reaction was linear with respect to the amount of cellular protein and the incubation time for all the different cell types.

2.4. Nucleotide, nucleoside and base analysis by HPLC

All determinations of metabolite concentrations were performed using high-performance liquid chromatography (HPLC). The equipment used was Merck–Hitachi chromatograph linked to 1050 series diode array detector (Hewlett–Packard) and type 507A on-line radiodetector (Berthold). Radioactive peaks of ATP/ADP and AMP collected from the APRT assay were counted using a Packard 1600-TR liquid scintillation analyser to determine the extent of adenine incorporation. The reversed-phase method used for determination of ATP, ADP, AMP, adenosine, inosine, hypoxanthine, xanthine, uric acid in the incubation medium, cell extracts and APRT enzyme reaction has been described in detail previously [17,18].

2.5. Statistical analysis

Values are presented as mean ± standard error of the mean (S.E.M.). Statistical comparison of different groups was performed using one-way analysis of variance (parametric or non-parametric Kruskal–Wallis test where appropriate) followed by Dunn's test. A value of $P < 0.05$ was considered as a significant difference.

3. Results

Fig. 1 shows the relationship between the time and the amount of adenine incorporated into the nucleotide pool of rat endothelial cells at 10 μM adenine concentration in the medium. As may be seen, the relationship was linear for up to 30 min of incubation. Fig. 2 demonstrates the concentration effect of adenine in the incubation medium and the amount incorporated into the rat endothelial nucleotide pool. The data demonstrates that adenine incorporation is a high affinity process with half saturation constant of about 0.5 μM.

Adenine incorporation under normal physiological

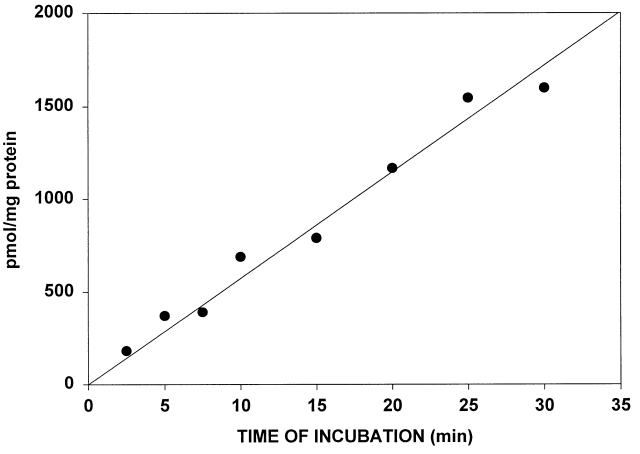

Fig. 1. Adenine incorporation into nucleotide pool in rat endothelium at different times of incubation. Cell cultures were incubated in the presence of 10 μM [^{14}C]adenine at 37°C in Hank's balanced salt solution (HBSS) for the time indicated. Values are the mean of duplicate experiments.

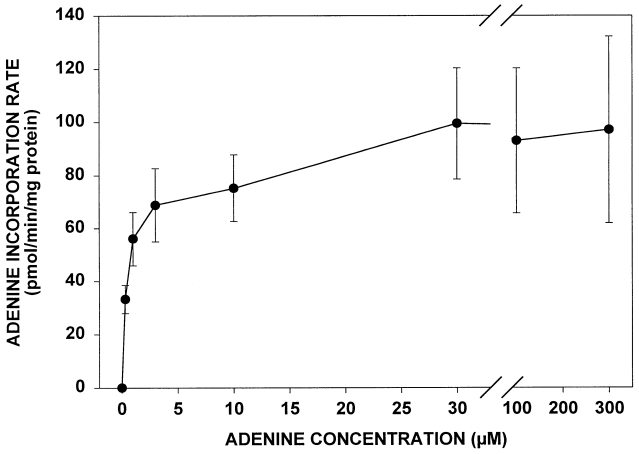

Fig. 2. Relation of adenine incorporation to its concentration in rat endothelium. Cell cultures were incubated in the presence of [^{14}C]adenine at 37°C in HBSS for 5 min. Values are mean ± S.E.M., $n = 4$–6.

concentration of phosphate and pH in rat, human and HMEC-1 endothelium is presented in Fig. 3. The rate observed in human microvascular cells was 25–35 times lower than in rat or in human transformed endothelium. The effect of different concentrations of ribose on adenine incorporation into the nucleotide pool of rat endothelial cells was evaluated within the concentration range 0.1 mM to 2.5 mM. No effect on adenine incorporation was observed (changes were less than 15% from control value). At 2.5 mM ribose adenine incorporation rate was 89.7 ± 12.5 pmol/min per mg protein as compared to 82.8 ± 11.1 pmol/min per mg protein without ribose under control conditions. The effect of inorganic phosphate on adenine incorporation into rat endothelial cells was evaluated within the concentration range of 0.5 to 4.8 mM. An increase in inorganic phosphate concentration exerted only a little effect on the rate of adenine incorporation (less than 20% difference from the control value). At 4.8 mM inorganic phosphate, adenine incorporation increased to 117.3 ± 14.0 pmol/min per mg protein. In the presence of both ribose and high inorganic phosphate, the rate of adenine incorporation was still not significantly different from control (112.4 ± 31.8 pmol/min per mg protein).

In human heart microvascular endothelial cells, the presence of 2.5 mM ribose exerted no effect and the rate was 3.0 ± 0.4 pmol/min per mg protein as compared to 3.1 ± 0.3 pmol/min per mg protein without ribose under control conditions. In the presence of both ribose and 4.8 mM inorganic phosphate, the rate of adenine incorporation was increased to 5.5 ± 0.5 pmol/min per mg protein ($P < 0.05$ vs. no ribose and phosphate).

In human microvascular cell line the presence of ribose did not exert any effect on the incorporation rate of adenine. The rate with 2.5 mM ribose was 117.9 ± 7.5 pmol/min per mg protein as compared to 115.1 ± 11.2 pmol/min per mg protein without ribose under control conditions. In the presence of 2.5 mM ribose and 4.8 mM inorganic phosphate the

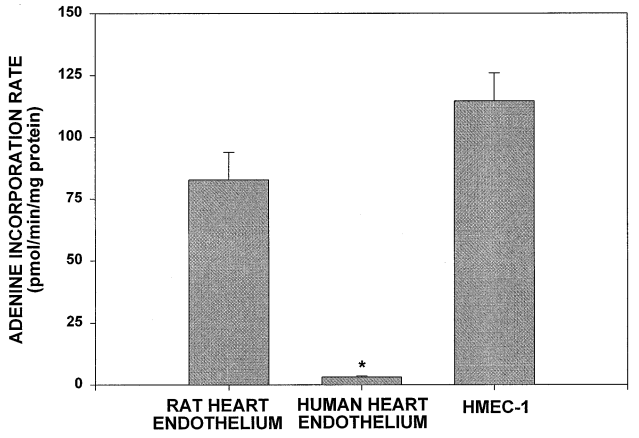

Fig. 3. Adenine incorporation rate in microvascular endothelial cells isolated from the rat and human heart and in a transformed human microvascular endothelial cell line (HMEC-1). Values represent mean ± S.E.M., $n = 5$–6. *$P < 0.05$ vs. rat endothelium and HMEC-1.

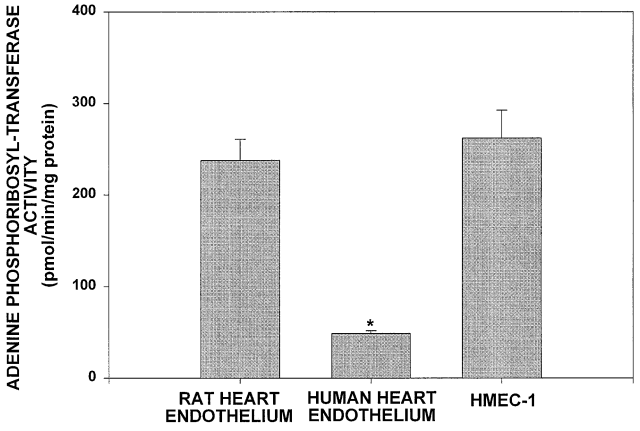

Fig. 4. Adenine phosphoribosyl transferase (APRT) activity in microvascular endothelial cells isolated from the rat and human heart and in a transformed human microvascular endothelial cell line (HMEC-1). Values represent mean ± S.E.M., $n = 6$–10. *$P < 0.05$ vs. rat endothelium and HMEC-1.

rate was even lower than control (90.2 ± 8.4 pmol/min per mg protein, $P < 0.05$ vs. no ribose and phosphate).

The APRT activity in human heart microvascular endothelial cells was found to be five times lower than in rat endothelial cells (Fig. 4). The activity for HMEC-1 cells was found to be comparable to the rate found in rat endothelial cells and again five-fold greater than the rate for normal human microvascular endothelial cells.

4. Discussion

This study demonstrated prominent differences in adenine incorporation rate between human and rat heart microvascular endothelial cells and between primary cultured human cells and transformed cell line. Adenine incorporation rate in human cells was only about 3% of the rate in rat endothelium. On the other hand, the transformed human microvascular endothelial cell line demonstrated a markedly increased adenine incorporation. Our measurements of APRT activity in the different cell types indicates that these differences could result from low activity of APRT in normal human cells. Nucleotide synthesis from adenine is thus inefficient in normal human endothelium, and an attempt to accelerate adenine incorporation in human endothelium could be beneficial for cell metabolism and function following oxidative stress or ischaemia which would result in nucleotide pool depletion.

Adenine reutilisation rate may be affected not only by APRT activity, but also by a number of other factors such as PRPP supply and membrane adenine transport. Our findings presented here, along with those of other authors, show large discrepancies between the activity of APRT measured in homogenates and the actual rate of reutilisation in intact cell models [19,20]. Membrane transport rate of adenine does not seem to be a limiting step of this process [21,22]. In erythrocytes and cardiomyocytes adenine reutilisation could be accelerated several-fold by the addition of ribose, a substrate for PRPP synthesis [2,19,23]. However, our study demonstrated that a supply of ribose or increasing phosphate concentration did not increase the rate of adenine reutilisation in rat endothelium. It is possible that this is the result of a high flux through the pentose phosphate pathway in endothelial cells and optimal supply of ribose moiety for nucleotide synthesis. The other factor which may affect the rate of adenine incorporation is inorganic phosphate, which is a potent activator of PRPP synthetase [5]. No changes in adenine nucleotide synthesis in rat endothelium with increased inorganic phosphate in the medium suggests a high flux through this reaction under baseline conditions, which is capable of providing an optimal supply for adenine reutilisation. Consequently, the concentration of adenine was in fact the only factor which controlled the rate of adenine incorporation in rat endothelial cells. It appears from the relation between adenine concentration and the rate of adenine incorporation presented in this study that the affinity is very high, comparable to the kinetic properties of the isolated enzyme [24].

Comparison of the rate of adenine reutilisation in endothelium reported in this study with the data published for cardiomyocytes [19] suggests that the rate under baseline conditions is much lower in cardiomyocytes than in endothelium. However, it has been shown that the addition of ribose caused an acceleration of adenine reutilisation in cardiomyocytes [11]. Consequently, with a supply of ribose, the rate could be similar in rat cardiomyocytes and endothelial cells.

In the present study, the rate of adenine reutilisation in human microvascular endothelial cells was 25 times smaller and the APRT activity was five times smaller than in rat endothelium. No effect of ribose was seen, this is in accordance with a previous study where adenine incorporation was evaluated in human vein endothelial cells [25]. Unlike in the rat endothelium, some increase in adenine incorporation was observed in the presence of ribose and phosphate in human microvascular cells, suggesting that PRPP concentration is below optimum. This may be responsible for the greater discrepancy between APRT activity and the rate of adenine incorporation.

Adenine incorporation pathway was found to be regulated in human cells, depending mainly on cell growth rate. In rapidly dividing trophoblast cells isolated from the first trimester placentas, the rate of adenine incorporation was tenfold greater than in the cells isolated in the third trimester [26]. This is in accordance with our observations indicating that

APRT activity and adenine incorporation rate is increased in rapidly growing transformed human endothelial cells. High APRT activity seems to be an isolated difference of nucleotide metabolism between human microvascular cell and HMEC-1 cell line, as we have not observed major differences in adenosine kinase or deaminase activities, purine nucleoside phosphorylase activity, or AMP-deaminase activity between both types of cells (unpublished observations). All types of cells were treated in a very similar way under culture conditions so it is unlikely to be a factor affecting the differences we observed. In addition, lactate dehydrogenase activity was comparable in the rat and human cells (not shown). The mechanism by which adenine incorporation could be increased in the normal human endothelial cells is an important challenge, since this would allow more efficient regulation of the nucleotide pool in human endothelial cells and all related physiological processes.

Acknowledgements

We gratefully acknowledge the support of the British Heart Foundation (Grant no. PG-96/194) for this work.

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